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(54) Enhanced growth of lactic acid bacteria in milk

(57) The present invention pertains to a growth medium adapted for the growth of a variety of different lactobacilli, which is based on milk and which is supplemented by at least four amino acids, ribonucleosides and iron. In particular, the present invention relates to the use of said novel medium for the cultivation of a variety of different *Lactobacilli*, preferably *Lactobacilli* belonging to Johnson's group A and B, especially for the production of dairy products.

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Description

[0001] The present invention pertains to a novel medium adapted for the growth of *Lactobacilli*, which comprises a milk-derived base supplemented by at least four amino acids, ribonucleosides and iron. In particular, the present invention relates to the use of said novel medium for cultivating a variety of different *Lactobacillus* strains, e.g. *L. johnsonii, L. acidophilus, L. gallinarum* for the preparation of dairy products.

[0002] Lactic acid bacteria have been used since long for the production of a variety of food material, such as yoghurt, cheese, curd etc. In addition to their general usage in the food industry for fermentation purposes quite recently some strains belonging to the genus *Lactobacillus* or *Bifidobacteria* have attracted a great deal of attention duė to probiotic, properties attributed to them. Consequently, there was a desire to improve cultivation conditions so as to be able to maximise the output of the microbial biomass.

[0003] One shortcoming of lactic acid bacteria with regard to their preparation on a large scale and their applicability, is to be seen in their different nutritional requirements. In this context, already different strains belonging to one specific genus or species require different media for optimal growth, which makes a standardised production of microbial biomasses complicated and cumbersome. Such, in producing a biomass of different strains of the genus *Lactobacillus*, a variety of different media have to be utilised, each of which does, however, only fulfil the nutritional needs of one particular strain, while not providing for a sufficient growth of other *Lactobacillus* strains therein.

[0004] A medium often utilised for cultivating lactic acid bacterial strains is cow milk. On the one hand, this medium provides a complex natural environment and its fermentation products, e.g. yoghurt, may be directly used as food material. Yet, this medium has proved to only support the growth of a limited number of strains of lactic acid bacteria. For example, *Lactobacilli* of the Johnson's group A and B have been demonstrated to be essentially unable to proliferate and grow in milk, which makes this medium useless for said strains.

[0005] In some cases, bacterial growth could be improved when substances of an undefined and very complex composition, such as yeast extract or peptones of various origins, had been added to the milk. However, these additional components can often cause an off-flavour with the result that cultures growing in a medium supplemented in such a way may not be used for industrial manufacture of dairy products. Moreover, the costs involved and the sometimes varying results in repeatability of achievable bacterial counts, makes them unsuitable for a commercial manufacture of microbial strains.

[0006] In view of this, a problem of the present invention is to provide a medium which supports the growth of *Lactobacillus* strains while avoiding the shortcomings of the state of the art.

[0007] This problem has been solved by providing a medium for the growth of *Lactobacillus* strains, which comprises a milk-derived base that has been supplemented by at least four aminoacids, ribonucleosides and iron.

[0008] According to a preferred embodiment, the amount of the ribonucleotide precursors (i.e. free bases, ribonucleosides, deoxyribonucleosides) to be added to the medium lies in the range from about 10 to about 500 mg/ 1 medium each, preferably from about 10 to about 100 mg/ 1 medium.

[0009] According to yet another preferred embodiment iron is added to the medium in an amount of from about 50 - 100 mg/ 1 milk.

[0010] Moreover, the medium is supplemented by at least four amino acids, which may be any existing amino acid available to the skilled person. The amount of amino acids to be added to the milk base lies in the range of from about 10 to about 200 mg / 1, preferably from about 50 to about 100 mg/ 1 milk. Yet, according to a preferred embodiment, the amino acids are selected from the group consisting of cysteine, alanine, serine and isoleucine, which are found to particularly improve the growth conditions for *Lactobacilli*.

[0011] According to yet another preferred embodiment the medium may also be supplemented by compounds providing a reducing activity, such as ascorbic acid, vitamin E, tocotrienol, ubiquinol, β-carotene and other carotinoids, rosemary compounds (e.g. camosol) and other flavonoids, and other sulphur containing antioxidants including glutathione, lipoic acids, N-acetylcysteine or compounds bearing sulfhydryl groups, cysteine or thioglycollic acid, or mixtures thereof. With respect to its use of an amino acid, cysteine is preferred as such a compound providing reducing activity.

[0012] The milk-derived base to be included in the medium may be milk in all of its variations, such as whole or partially de-fatted milk, skim milk or UHT milk or may be prepared from dried milk powder by addition of water. The fluid milk base may be used as such or other well known components may be added, such as e.g. water, to dilute the milk to a desired degree.

[0013] In the figures,

[0014] **Figure 1** shows a comparison between RABIT curves obtained after 24 hours incubation for *L. johnsonii* La1 (NCC 533) in 10% skim and whole fat UHT milk supplemented with 1% yeast extract and a mixture of four ribonucleosides, four amino acids and ferrous sulphate; (1) whole UHT milk + four ribonucleosides + four amino acids; (2) whole fat UHT milk + yeast extract; (3) skim + yeast extract; (4) skim milk + four ribonucleosides + four amino acids + ferrous sulphate; (5) skim milk + adenosine and guanosine + four amino acids + ferrous sulphate.

[0015] **Figure 2** shows the effect of a supplementation of a 10% skim and whole fat UHT milk with four ribonucleo-

sides, four amino acids and ferrous sulphate on the growth of *L. gallinarum* DSM 33199^T; (1) 10% skim milk; (2) 10% skim milk + four amino acids + four ribonucleosides + ferrous sulphate; (3) whole fat UHT milk; (4) whole fat UHT milk + four amino acids + four ribonucleosides + ferrous sulphate.

[0016] During the extensive studies leading to the present invention it has been found that various parameters seem to be responsible for the growth of *Lactobacilli* in a milk-based medium.

[0017] Cow's milk is known to have a specific content of ribonucleotides, which varies depending on the season and country of production. The purine derivatives account for just a low amount, while about more than 95 % of the ribonucleotides in milk is represented by orotic acid, that is used as a pyrimidine precursor by bacterial cells. The low content of adenine and guanine nucleotides in milk negatively affects the bacterial growth with the proviso of some strains that may perform a *"de-novo"* synthesis of DNA and RNA precursors, such as *L. casei* and *L. plantarum*. However, even inhibitory effects have been observed in some cases when adding purine derivatives to milk.

[0018] Since the inability of some *Lactobacillus* strains, such as *L. johnsonii, L. gasseri, L. crispatus, L. amylovorus, L. gallinarum* and *L. acidophilus,* to reproduce at high density in milk a combination of different chemicals was studied which were hypothesised to be capable to replace growth stimulatory substances of undefined composition.

[0019] In order to find the identity of other putative stimulatory substances, several trials were performed with ribonucleotide precursors, that is free bases (adenine, guanine, cytosine, thymine, uracil), ribonucleosides (adenosine, cytidine, uridine, guanosine) and 2'-deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and thymidine). They were supplemented to milk as concentrated alkaline or neutral solutions at different concentrations.

[0020] An addition of ribonucleosides improved the growth conditions for *lactobacilli* in milk, with adenosine and guanosine showing the strongest effect. This finding confirmed the hypothesis that the low level of purines in milk obviously negatively affects bacterial growth therein. Generally it was found that the high content of orotic acid represents a stimulatory factor for *Lactobacillus* growth, allowing the synthesis of pyrimidine bases. No significant differences in pH-values were detected by the addition of free bases and deoxyribonucleosides between aerobic and anaerobic conditions. More than 1 log improvement in bacterial growth was observed, indicating a positive effect concerning particularly ribonucleosides in anaerobic environment.

[0021] The best improvements for increasing the number of *Lactobacilli* by addition of ribonucleosides and the strongest acidification levels, respectively, were achieved by the addition of adenosine, guanosine, and/or cytidine and uridine in an amount of about 0.1 g/l each. Though this mixture on its own revealed the ability to support *L. johnsonii, L. acidophilus,* and *L. gallinarum* growth at levels comparable to those achieved by the addition of yeast extract (see **Figures 1, 2** and **Table 3**) no remarkable positive effects were observed with other *Lactobacillus* strains, from the species *L. amylovorus, L. crispatus* and *L. gasseri.*

[0022] Upon addition of free bases to milk (adenine, cytosine, uracil, thymine and guanine) in replacement of ribonucleosides as supplements, similar results were obtained. However, in that case the strains tended to show a requirement for magnesium and aspartic acid.

[0023] In addition, several trials were conducted by supplementing milk with different 2'-de-oxynucleosides which brought about an increase of the viable cell number of merely some particular strains.

[0024] In spite of the above finding that none of the mentioned chemicals was able to support bacterial growth for a plurality of different bacterial strains at a high level if added alone to the milk, it has surprisingly been found that a combination consisting of amino acids, ribonucleosides and iron (e.g. in the form of iron sulphate) does actually promote the growth of different *Lactobacillus* species. In experiments, in which the number of the different compounds in the above combination mixture was reduced to a minimum it could be seen that the least number for each of the compounds specified to be added to the milk is at least two ribonucleosides, preferably adenosine and guanosine, four amino acids and iron. This mixture was able to improve growth of a variety of different *Lactobacillus* strains, such as those of the Johnson's group, with cell counts and a final pH comparable to that obtained by addition to the milk of yeast extract or peptones.

[0025] Moreover, the addition of iron to a mixture consisting of the medium supplemented with a combination of the above chemicals has been found to even improve the results obtained. This finding may be explained such that despite its rich composition, milk presents a strong deficiency in iron which is complexed as lactoferrin and is therefore unavailable for any microorganism growing therein.

[0026] Thus, the best results were obtained by addition of adenosine, guanosine, and/or cytidine and uridine in an amount of 0.1 g/l each, alanine, serine, isoleucine, cysteine (0.05 g/l each) and $FeSO_4$ (0.1 g/l).

[0027] The fact that both skim and whole fat UHT milk gave optimal results when supplemented with the combination of the above compounds, led to the assumption that the fatty components of milk had no role in stimulating *Lactobacillus* growth as well as sterilisation treatments (UHT) did not negatively affect the potential of milk to support bacterial development.

[0028] The following examples illustrate the invention without limiting the same thereto.

Examples

Bacterial strains and culture conditions:

[0029] *L. johnsonii* strains ATCC 33200^T, La1 (NCC 533), ATCC 11506 (formerly known as *L. acidophilus* R-26), ATCC 332, DSM 20553, , *L. acidophilus* ATCC 4356^T, LalO (NCC 90), *L. gasseri* DSM 20243^T, *L. crispatus* DSM 20531^T, *L. amylovorus* DSM 20584^T and *L. gallinarum* DSM 33199^T were propagated in MRS (Difco) broth or agar at 37°C. Skim milk (Difco) 10% w/v in sterile water and whole fat UHT milk (Parmalat, Italy) were used to perform the growth assays. The milk tubes were 1% inoculated from an overnight MRS culture washed twice and finally re-suspended with the same amount of sterile distilled water in order to avoid nutrient transfer via the medium.

Incubation parameters:

[0030] Milk tubes were incubated both aerobically in a thermostat (Sorvall Heraeus) at 37°C for 24 hours, and anaerobically in an anaerobic incubator (model 1024, Forma Scientific, USA) at 37°C for 24 hours.

Milk supplementation:

[0031] The chemicals supplemented to the milk were added as concentrated solutions prepared according to the Merck Index instructions. The final pH of milk was adjusted to 6.8 after supplementation using 4N NaOH. The initial pH of the 10% skim milk solution and whole fat UHT milk was 6.8 and 6.7, respectively.

Bacterial growth estimation:

[0032] The growth results were estimated by cell counts and a final pH measurement was performed after 24 hours incubation at 37°C.

[0033] Rapid Analysis of Bacterial Impedence Technique (RABIT) (Don Whitley Scientific, West Yorkshire, UK) was used to perform trials with skim and whole UHT milk for 24 hours at 37°C.

[0034] The experiments were performed using the listed 11 strains of all six species of the Johnson's group A and B, including the type strain of *L. johnsonii* ATCC 33200 and *L. johnsonii* La1 (NCC 533) in order to determine its nutritional requirements in milk. The results led to the identification of some chemicals able to reproduce the positive effects of yeast extract and other substances of chemically undefined composition on bacterial growth in milk.

[0035] The strains under investigation were demonstrated not to be able to grow in both 10% skim and whole fat UHT milk. The results, summarised in **Table 1** for *L. johnsonii*, indicate that a moderate acidification of these natural media after 24 hours incubation occurred, resulting in less than 1 log increase of final viable cell numbers even if the incubation was performed under anaerobic conditions. The same behaviour in both skim and whole fat UHT milk was observed also for the type strains of *L. gasseri, L. amylovorus, L. crispatus, L. acidophilus* and *L. gallinarum.*

[0036] The supplementation of skim milk with 1% v/v yeast extract (Adsa, Italy) resulted in a 2 log improvement in the viable cell number. This result could be confirmed using whole fat UHT milk. After 24 hours incubation a final pH of 4.0 was obtained with yeast extract addition (**Table 1**).

[0037] The final yeast extract concentration required for optimal bacterial growth varied between 0.1 and 1.0% v/v . The development of off-flavours and colour changes can be observed in fermented dairy products supplemented with this substance.

Table 1

Final pH and cell counts after 24 hours incubation of *L. johnsonii* La 1 (NCC 533) in 10% skim / UHT milk and after supplementation with 1% yeast extract. All results were reconfirmed for the other investigated *L. johnsonii* strains, with exception of strain ATCC 332, which did not show growth even with yeast extract addition.

	10 % skim milk		UHT	Skim + yeast extr.		UHT + yeast extr.
	pH	CFU/ml	pH	pH	CFU/ml	pH
AEROBIOSIS						
Initial value	6.8	$1.0x10^7$	6.7	6.8	$1.0x10^7$	6.7
24 hours	6.0	$1.8x10^7$	6.3	3.9	$3.0x10^9$	4.0

Table 1 (continued)

AEROBIOSIS						
ANAEROBIOSIS						
Initial value	6.8	$1.0x10^7$	n.d.	6.8	$1.0x10^7$	n.d.
24 hours	5.9	$7.0x10^7$	n.d.	3.8	$3.2x10^9$	n.d.

[0038] A mixture of 19 amino acids (alanine, glycine, histidine, lysine, phenylalanine, proline, serine, threonine, cysteine, arginine, aspartic acid, asparagine, glutamic acid, isoleucine, leucine, methionine, tyrosine, tryptophane and valine) was added to skim milk (final concentration 0.05 g/l v/v of each amino acid) producing a positive effect on *L. johnsonii* development which was almost comparable to acidification levels after yeast extract addition. A final pH of 4.1 was measured after amino acids supplementation but the cell count was still not satisfactory (4x10E+08 cfu/ml).

[0039] In order to determine those amino acids having an essential role for *L. johnsonii* growth in milk, the "omission technique" (Reiter, B. & Oram, J.D, J. Dairy Res, **29** (1962), 63-77) was applied by culturing the strain ATCC 33200^T in skim milk, adding four ribonucleosides + ferrous sulphate (positive control), supplemented with the mixture of 19 amino acids described above deprived of one particular component at each given time. Rapid Analysis of Bacterial Impedence Technique (RABIT) allowed the identification of four amino acids (cysteine, alanine, serine and isoleucine) that showed excellent results. The latter three were thought to be stimulants for the tested strain when exogenously added to the milk.

[0040] The strongest role, among the identified amino acids, was attributed to cysteine, confirming that this absence of cysteine or cystine in milk, may negatively affect bacterial development. The role of SH groups seems not completely replaceable by anaerobiosis. The absence of oxygen realized by anaerobic incubation of the *L. johnsonii* cultures did not allow the achievement of the same growth results obtained when cysteine was supplemented to skim or whole fat milk.

[0041] The pH measurement revealed a value of pH 4.3 in absence of cysteine under anaerobic conditions against a pH of 3.9 obtained in the presence of this compound under aerobic conditions. However, the removal of cysteine resulted in a more significant loss of the viable cell numbers in an aerobic rather than an anaerobic environment. When *L. johnsonii* was cultured under aerobic conditions, a solution of thioglycollic acid (final concentration 0.5% v/v) revealed its ability to replace cysteine, resulting in high cell counts of more than 1.0x10E+09 cfu/ml.

[0042] In spite of the stimulatory actions due to the four cited amino acids (cysteine, alanine, serine and isoleucine) an unexpected negative effect was observed for all other 15 aminoacids (for an example see **Figure 3**).

Table 2

Final pH after 24 hours incubation at 37°C of *L. johnsonii* La1 (NCC 533) in 10% skim milk (initial pH 6.8) supplemented with 0.1 g/l (v/v) free bases (adenine, cytosine, guanine,uracil and thymine), ribonucleosides (adenosine, cytidine, guanosine, uridine) or deoxyribonucleosides (2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, 2'-deoxyuridine, thymidine).		
Chemical	**Aerobiosis**	**Anaerobiosis**
Free bases	5.9	5.8
Ribonucleosides	6.3	5.6
Deoxyribonucl.	5.8	5.9

free bases:	adenine, cytosine, guanine, uracil and thymine
ribonucleosides:	adenosine, cytidine, guanosine, uridine
deoxyribonucleosides:	2-deoxyadenosine, 2-deoxyguanosine, 2-deoxycytidine, 2-deoxyuridine, thymidine.

[0043] Similar results were obtained for other strains, e.g. *Lb. johnsonii* ATCC 33200^T

Table 3.

Final pH after 24 hours incubation at 37°C of *L. johnsonii* La 1 (NCC 533) and other *Lb. johnsonii* strains in 10% skim milk, under aerobic and anaerobic incubation conditions, and whole fat UHT milk supplemented with four ribonucleosides, four aminoacids and ferrous sulphate.						
	NCC 533	**ATCC 33200**	**DSM 20553**	**ATCC 332**	**ATCC 11506**	**DSM 33199**
Aerobiosis						

Table 3. (continued)

	NCC 533	ATCC 33200	DSM 20553	ATCC 332	ATCC 11506	DSM 33199
10% skim milk	4.0	5.4	4.2	5.1	5.3	4.5
whole fat UHT milk	3.9	5.5	4.8	6.2	5.4	4.5
Anaerobiosis						
10% skim milk	4.2	Nd	Nd	nd	Nd	nd
whole fat UHT milk	nd	Nd	Nd	nd	Nd	nd

[0044] Similar results were obtained for other strains.

Claims

1. A medium for growing lactobacilli comprising a milk-derived base, characterised in that at least four amino acids, ribonucleosides and iron are added in an amount sufficient to promote growth of Lactobacilli therein.

2. The medium according to claim 1, wherein the amount of ribonucleosides is in the range of from about 1 to about 500 mg/l, preferably from about 10 to about 100 mg/l.

3. The medium according to claim 1 or claim 2, wherein the ribonucleosides are adenosine or guanosine.

4. The medium according to any of the preceding claims, which contains iron in an amount ranging from about 10 to about 200 mg/l, preferably from about 50 to about 100 mg/l milk.

5. The medium according to any of the preceding claims, wherein the amino acids added are preferably cysteine, alanine, serine and isoleucine, in an amount ranging from about 10 to about 200 mg/l, preferably from about 50 to about 100 mg/l milk.

6. The medium according to any of the preceding claims which contains compounds providing reducing activity.

7. The medium according to claim 6, wherein the compound providing reducing activity is selected from the group consisting of cysteine, thioglycollic acid, ascorbic acid or mixtures thereof.

8. The use of a medium according to any of the preceding claims for cultivating lactobacilli belonging to Johnson's group A and B.

9. Use of a medium according to any of the claims 1 to 7 for the production of fermented or non-fermented dairy products.

Figure 1

(1) whole UHT milk + four ribonucleosides + four amino acids;

(2) whole fat UHT milk + yeast extract;

(3) skim + yeast extract;

(4) skim milk + four ribonucleosides + four amino acids + ferrous sulphate;

(5) skim milk + adenosine and guanosine + four amino acids + ferrous sulphate.

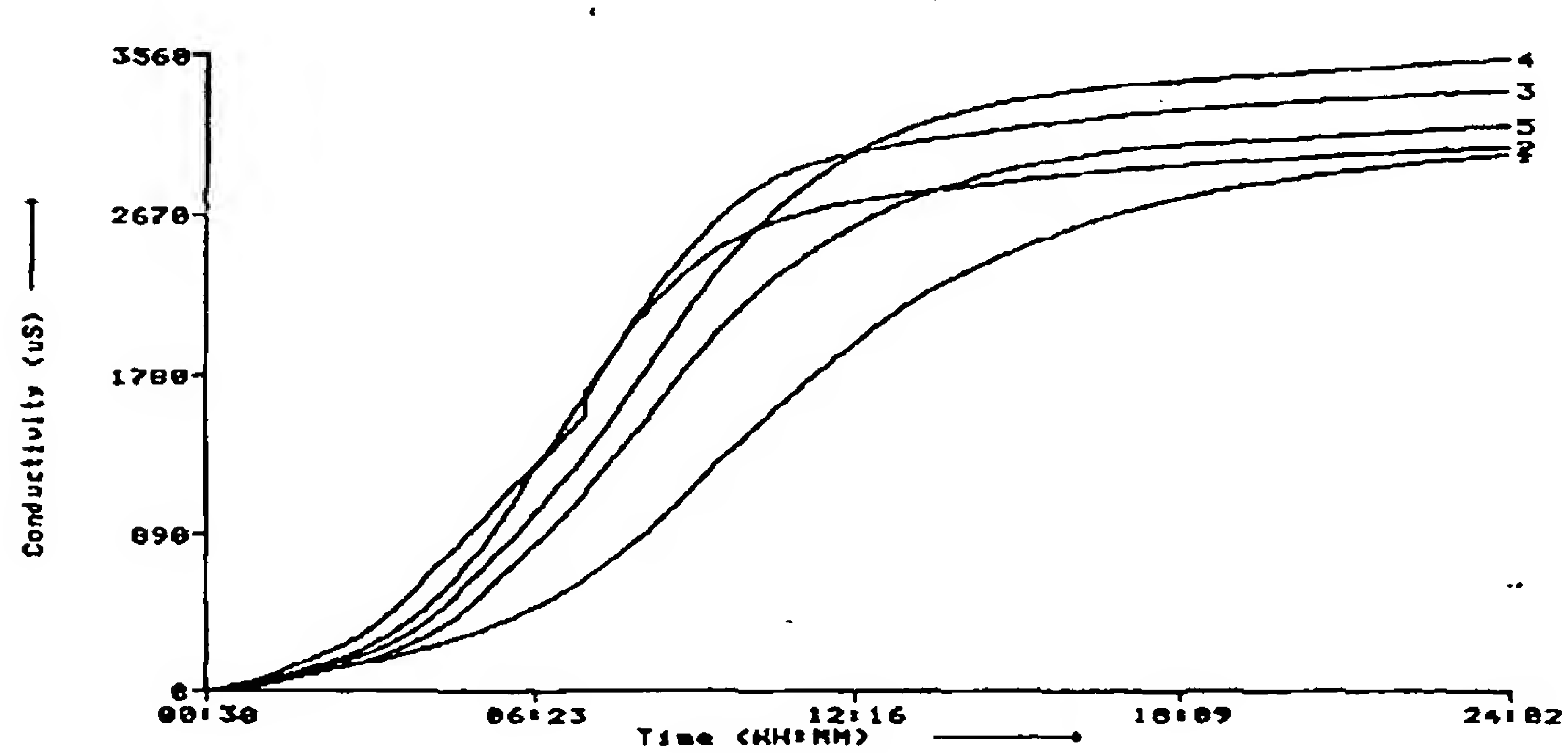


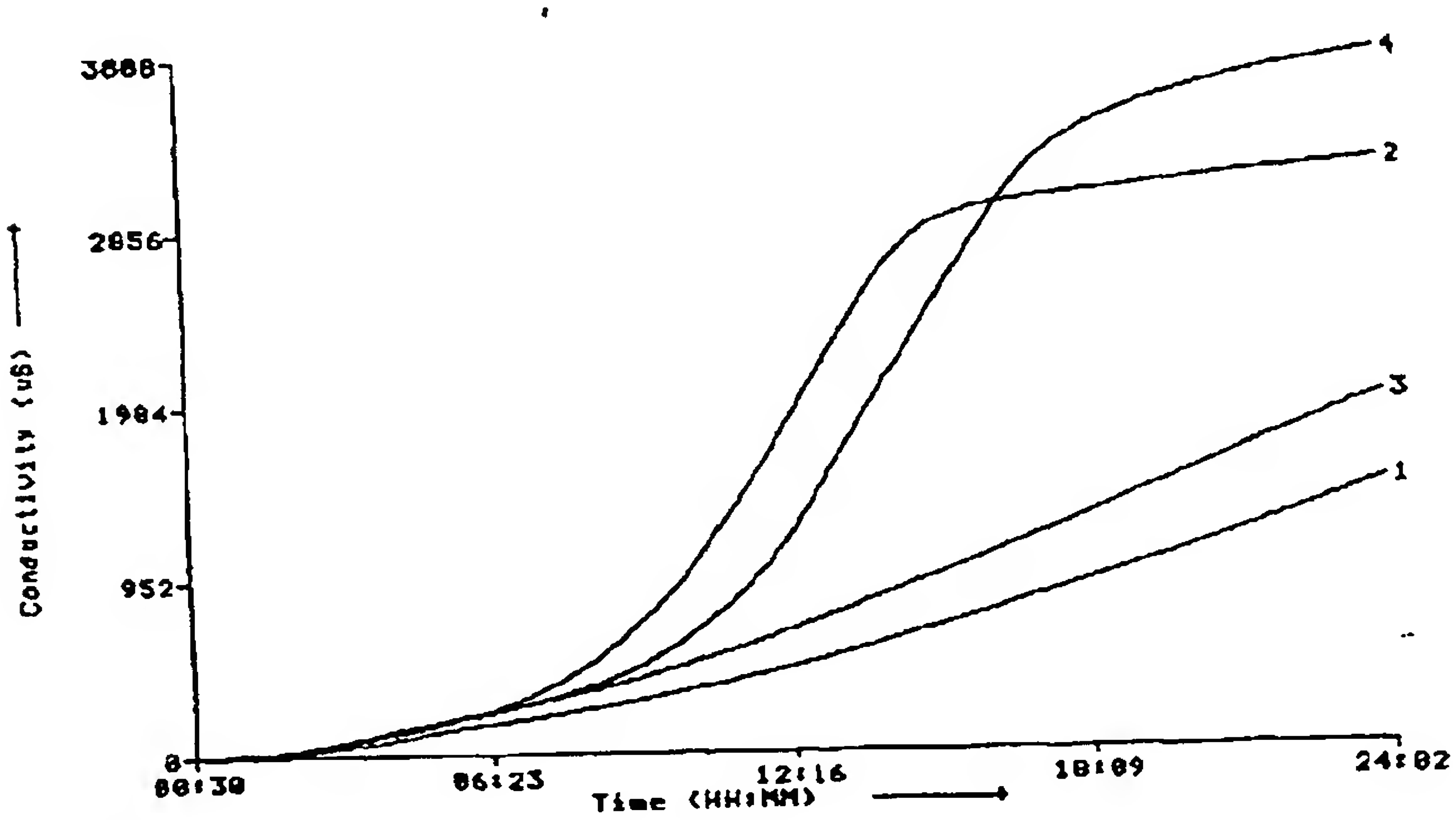
Figure 2

(1) 10% skim milk;

(2) 10% skim milk + four amino acids + four ribonucleosides + ferrous sulphate;

(3) whole fat UHT milk;

(4) whole fat UHT milk + four amino acids + four ribonucleosides + ferrous sulphate.



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EUROPEAN SEARCH REPORT

Application Number
EP 99 10 8717

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	13 September 1999	Panzica, G

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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